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Lactose Transport System of *Streptococcus thermophilus*

THE ROLE OF HISTIDINE RESIDUES*

(Received for publication, November 6, 1991)

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The lactose transport protein (LacS) of *Streptococcus thermophilus* is a chimeric protein consisting of an amino-terminal carrier domain and a carboxyl-terminal phosphoenolpyruvate:sugar phosphotransferase system (PTS) IIA protein domain. The histidine residues of LacS were changed individually into glutamine or arginine residues. Of the 11 histidine residues present in LacS, only the His-376 substitution in the carrier domain significantly affected sugar transport. The region around His-376 was found to exhibit sequence similarity to the region around His-322 of the lactose transport protein (LacY) of *Escherichia coli*, which has been implicated in sugar binding and in coupling of sugar and H⁺ transport. The H376Q mutation resulted in a reduced rate of uptake and altered affinity for lactose (β -galactoside), melibiose (α -galactoside), and the lactose analog methyl- β -D-thiogalactopyranoside. Similarly, the extent of accumulation of the galactosides by cells expressing LacS(H376Q) was highly reduced in comparison to cells bearing the wild-type protein. Nonequilibrium exchange of lactose and methyl- β -D-thiogalactopyranoside by the H376Q mutant was ~2-fold reduced in comparison to the activity of the wild-type transport protein. The data indicate that His-376 is involved in sugar recognition and is important, but not essential, for the cotransport of protons and galactosides.

The carboxyl-terminal domain of LacS contains 2 histidine residues (His-537 and His-552) that are conserved in seven homologous IIA protein(s) (domains) of PTSs. P-enolpyruvate-dependent phosphorylation of wild-type LacS, but not of the mutant H552Q, was demonstrated using purified Enzyme I and HPr, the general energy coupling proteins of the PTS, and inside-out membrane vesicles isolated from *E. coli* in which the lactose transport gene was expressed. The His-537 and His-552 mutations did not affect transport activity when the corresponding genes were expressed in *E. coli*.

The lactose transport protein of *Streptococcus thermophilus* consists of an amino-terminal hydrophobic carrier domain that is homologous to the melibiose carrier protein of *Escherichia coli* and a carboxyl-terminal hydrophilic domain that is homologous to IIA proteins or IIA protein domains of

the phosphoenolpyruvate:sugar phosphotransferase system (Poolman *et al.*, 1989; Liau *et al.*, 1991). In earlier reports, the IIA protein and IIA protein domains were designated Enzyme III and Enzyme III domains, respectively. The IIA protein domain of LacS contains 2 histidine residues (His-537 and His-552) that are conserved in the homologous PTS¹ proteins (Fig. 1) (Poolman *et al.*, 1989). His-552 of LacS corresponds to His-91 of the IIA^{Glc} protein of *E. coli*, which has been shown to be phosphorylated by HPr~P (Dörschug *et al.*, 1984). The lactose transport protein (LacS) is a secondary active transport system that catalyzes the uptake of various α - and β -galactosides in symport with a proton as well as exchange of galactosides.² The heterologous exchange of lactose for galactose most likely occurs during metabolism of lactose by *S. thermophilus* and *Lactobacillus bulgaricus* (Poolman, 1990).

Although the overall similarity between the carrier domain of LacS and the lactose carrier (LacY) of *E. coli* is not significant (Poolman *et al.*, 1989), a region (H376/E379) in LacS can be identified that is similar to the H322/E325 region in LacY (Fig. 1). Evidence has been presented indicating that His-322, Glu-325 (putative helix X), and Arg-302 (putative helix IX) of LacY are in close contact with each other and that these residues could form a "charge relay" system that participates in the coupled transmembrane movement of protons and galactosides (Püttner *et al.*, 1986, 1989; Carrasco *et al.*, 1986, 1989; Menick *et al.*, 1987; Lee *et al.*, 1989). Recently, the requirement of an ionizable histidine residue at position 322 in LacY for galactoside/proton symport has been questioned (King and Wilson, 1989a, 1989b, 1990; Franco and Brooker, 1991). It has been shown that some His-322 mutants of LacY, which do not build up a galactoside concentration gradient, catalyze galactoside-dependent proton transport (King and Wilson, 1989a, 1989b, 1990). Moreover, the H322N mutant of LacY still accumulates lactose against a concentration gradient, although the levels of accumulation are low compared to those of the wild-type strain (Franco and Brooker, 1991).

In this study, we assess the role of the histidine residues in the carrier and IIA protein domains of the lactose transport protein of *S. thermophilus*. The results indicate that His-552 is phosphorylated by HPr(His~P) from *Bacillus subtilis*, but that the conserved histidine residues in the IIA protein domain of LacS are not essential for transport. His-376 in the carrier domain of LacS could serve a role similar to that of His-322 in LacY. The similarities and differences between the proposed active-site residues of LacS and LacY are discussed.

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§ Supported by a fellowship from the Royal Netherlands Academy of Arts and Sciences. To whom correspondence should be addressed. Tel.: 31-50-632170; Fax: 31-50-635205.

¹ The abbreviations used are: PTS, phosphoenolpyruvate:sugar phosphotransferase system; TMG, methyl- β -D-thio-galactopyranoside.

² C. Foucaud and B. Poolman, submitted for publication.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids

Bacterial strains are described in Table I. The cells were grown aerobically in Luria broth at 37 °C (Sambrook *et al.*, 1989) unless indicated otherwise. Plasmid pEKS8 containing a 4.1-kilobase *EcoRI* chromosomal DNA fragment of *S. thermophilus*, encoding the lactose transport protein (LacS), has been described (Poolman *et al.*, 1989, 1990). For the construction of pSKE8, the 4.1-kilobase *EcoRI* fragment of pEKS8 was ligated in the *EcoRI* site of pBluescript IISK+ (Stratagene). Plasmid pAVL1 is pACYC177 that carries a 9.5-kilobase *HindIII* chromosomal fragment of *Klebsiella pneumoniae* KAY2026, carrying the *nag* operon, including *nagE*, which codes for IICBA-N-acetylglucosamine (Vogler *et al.*, 1988).

Site-directed Mutagenesis

The mutagenic primers are listed in Table II. Site-directed mutagenesis was carried out by the method of Kunkel *et al.* (1987).

Mutations in pSKE8—Single-stranded uracil-containing DNA of pSKE8 was isolated from *E. coli* CJ236 (*dut*⁻, *ung*⁻)/pSKE8 after infection with helper phage M13KQ7 (Sambrook *et al.*, 1989). Closed-circular heteroduplex DNA with the desired mutations was synthesized *in vitro* as described (Kunkel *et al.*, 1987) and transformed to *E. coli* JM101 (*ung*⁺). Plasmid DNA was isolated from a number of transformants, and plasmids bearing the desired mutation(s) were identified by digestion with restriction enzymes for which a site was lost or created by the mutagenesis (see Table II). Subsequently, mutations were verified by nucleotide sequencing of double-stranded DNA using the dideoxy chain termination method (Sanger *et al.*, 1977) and a set of primers complementary to a region of *lacS* located 50–100 bases downstream or upstream of the mutation site. Each mutant was isolated independently at least twice.

Mutations in pEKS8—The 648-base pair *KpnI*-*EcoRI* fragment of pEKS8, comprising the carboxyl-terminal 323-base pair region of *lacS*, was ligated in the replicative form of M13mp18/M13mp19. Recombinant phage DNA was used to transfect *E. coli* CJ236; and single-stranded uracil containing M13mp18 or M13mp19 DNA, including the *KpnI*-*EcoRI* fragment, was isolated. Synthesis of the complementary strand and screening of mutants were carried out as described above. The entire region of *lacS* was sequenced. Subsequently, the *KpnI*-*EcoRI* fragment of pEKS8 was exchanged for the corresponding fragment containing the desired mutation that was isolated from the replicative form of the M13 DNA.

Preparation of Cell Suspensions

Overnight cultures or exponentially grown cells ($A_{660} = 0.4$ – 0.8) were harvested by centrifugation, washed twice, and resuspended to a final protein concentration of 20–80 mg/ml in 50 mM potassium phosphate (pH 6.5), 5 mM magnesium sulfate (KPM buffer). Measurements of the membrane potential and analysis of the effects of ionophores (valinomycin, nigericin) on transport were performed with

E. coli cells that were treated with EDTA essentially as described previously (Sarkar *et al.*, 1988). Concentrated cell suspensions were stored on ice until use.

Transport Assays

Transport experiments were performed at 20, 30, or 37 °C as specified in the figure legends.

Active Transport—Cells were diluted to a final protein concentration of 0.6–1.2 mg/ml in KPM buffer containing 10 mM D-lithium lactate as the electron donor. After 2 min of pre-energization in the presence of oxygen, radiolabeled substrate was added; and at appropriate time intervals, the uptake reaction was stopped by addition of 2 ml of ice-cold 100 mM LiCl. Cells were collected by filtration on a 0.45- μ m cellulose nitrate filter (Millipore Corp.) and washed with 2 ml of ice-cold LiCl.

Efflux and Exchange—Preloading of cells with sugars was achieved by incubation (overnight at 4 °C) of the concentrated washed cell suspensions with the appropriate concentration of radiolabeled galactoside in the presence of deoxyribonuclease I (20 μ g/ml). The next day, potassium azide and carbonyl cyanide *m*-chlorophenylhydrazone were added to final concentrations of 30 mM and 50 μ M, respectively; and the cells were incubated for another 2 h at room temperature essentially as described (King and Wilson, 1990). For efflux and exchange, 1- μ l aliquots of concentrated cell suspension (50–80 mg/ml) were diluted into 500 μ l of KPM buffer containing no substrates and unlabeled galactosides, respectively. The transport reaction was stopped by rapid filtration as described above.

Isolation of Membranes

For the isolation of inside-out membrane vesicles of *E. coli* T184, T184/pEKS8, T184/pEKS8(H552Q), T184/pEKS8(H537A), and T184/pEKS8(I548E/G556D), cells were lysed by a 2-fold passage through a French pressure cell (20,000 p.s.i.), and membranes were collected as described (Poolman *et al.*, 1983). The membranes were washed and resuspended to 20–30 mg of protein/ml in 10 mM Tris-HCl (pH 7.5) containing 0.5 mM phenylmethylsulfonyl fluoride.

Protein Purification

Enzyme I and HPr of *B. subtilis* and *E. coli* were purified as described previously (Reizer *et al.*, 1989, 1992).

Phosphorylation Assay

Inside-out membrane vesicles (5 mg/ml) were incubated in 50 mM Tris-HCl (pH 7.5) containing 5 mM MgCl₂, 0.1 mM dithiothreitol, 0.5 mM NaEDTA, purified HPr (0.5 mg/ml) and Enzyme I (0.35 mg/ml), and [³²P]P-enolpyruvate (Reizer *et al.*, 1984). The phosphorylation reaction was carried out at 37 °C for 15 min in a total volume of 15 μ l. The reaction was stopped by addition of an equal volume of Laemmli sample buffer (Laemmli, 1970), and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5%

TABLE I
Bacterial strains

Strain	Genotype	Ref.
DW1	<i>lacI</i> ⁺ , Δ <i>lac</i> (ZY), Δ <i>mel</i> (AB), <i>strA</i>	Wilson and Wilson (1987)
DW2	<i>lacI</i> ⁺ , Δ <i>lac</i> (ZY), <i>melA</i> ⁺ , Δ <i>melB</i> , <i>strA</i>	Wilson and Wilson (1987)
PPA209	DW2, <i>pts1001</i> <i>zfe::Tn10</i>	Postma ^a
T184	<i>lacI</i> ⁺ <i>O</i> ⁺ ZY(A), <i>rpsL</i> , <i>met</i> , <i>thr</i> , <i>recA</i> , <i>hsdR</i> , <i>hsdM</i> (F', <i>lacI</i> ⁺ <i>O</i> ⁺ Z ^{U118} (Y ⁺ A ⁺))	Teather <i>et al.</i> (1980)
LR2-162	<i>thi-1</i> , <i>hisG1</i> , <i>argG6</i> , <i>metB1</i> , <i>galT6</i> , <i>xyl-7</i> , <i>supE44</i> , <i>rpsL104</i> , Δ <i>phoA8</i> , <i>tonA2</i> , <i>ptsM</i> , <i>manI</i> , <i>man-162</i> , <i>asnB::Tn5</i> , F ⁻	Vogler <i>et al.</i> (1988)
LR2-167	LR2-162, <i>nagE</i>	Vogler <i>et al.</i> (1988)
LM1	LR2-162, <i>nagE</i> , <i>err-1</i>	Vogler <i>et al.</i> (1988)
JM101	<i>supE</i> , <i>thi</i> , Δ (<i>lac-proAB</i>), (F', <i>traD36</i> , <i>proAB</i> , <i>lacI</i> ⁺ Δ M15)	Yanisch-Perron <i>et al.</i> (1985)
CJ236	<i>dut-1</i> , <i>ung-1</i> , <i>thi-1</i> , <i>relA1</i> /pCJ105 (Cm ^R)	Kunkel <i>et al.</i> (1987)
HB101	<i>hsdS20</i> (r _B ⁻ m _B ⁻), <i>recA13</i> , <i>ara14</i> , <i>proA2</i> , <i>lacY1</i> , <i>galK2</i> , <i>rps</i> (sm ^R), <i>xyl-5</i> , <i>mtl-1</i> , <i>supE44</i> , λ^- , F ⁻	Boyer and Roulland-Dussoix (1969)

^a P. W. Postma, unpublished data.

TABLE II
Mutations in the lactose transport gene of *S. thermophilus*

The nucleotides changed by the site-directed mutagenesis are underlined. The nucleotide changes resulting in the desired amino acid substitution are indicated in boldface type. The last column indicates whether a new restriction was created or a restriction site was lost as a result of the mutagenic event(s).

Mutation	Sequence of mutagenic primer	Restriction analysis
H39Q	5'-TTGTGACA <u>ACTCAAT</u> TGTTTAAACAGGGGATCCAAAG	New <i>Bam</i> HI
H51Q	5'-GCAAAATAGTCAATACGTTCTATTAATC	Loss of <i>Sna</i> BI
H155Q	5'-CTTGCTCTAGATAGTCAAGAACGTGAA	New <i>Xba</i> I
H376Q	5'-AAACGGGAC <u>AA</u> CGGGATGAATCAC	New <i>Fok</i> I
H422Q	5'-ACAACACAAC <u>ACAG</u> TTTATCTTTAAACTTGGCAT	Loss of <i>Hind</i> III
H457Q	5'-AGCACGTCAAGCCAAAATTGTGGAAGAAATTGG	New <i>Bst</i> XI
H466Q	5'-TTGTTGAAGAGCTCGAACAACGCTTTAGCG	New <i>Sst</i> I
H502Q	5'-TCTCAAGTGTCAACGATGAACAATTTGCTTCA	New <i>Hinc</i> II
H537A	5'-TCCTACTCGCGCTGCAGTTGGTA	Loss of <i>Bst</i> XI
H537R	5'-TCCTACTCGCGCTGCAGTTGGTA	Loss of <i>Bst</i> XI
H552Q	5'-GTTCTTATCCAGGTTGGCATCGGTACCGTTAAACTT	New <i>Kpn</i> I
H612Q	5'-GTGCTTTCCAACTCGAGCAAAAAGTTG	New <i>Ava</i> I
I548E/G556D	5'-AGATGGTGTGCAAGTTCTTATCCACGTTGGCATCGATACAGTTAAAC	New <i>Cla</i> I- <i>Taq</i> I

polyacrylamide). The 32 P-labeled proteins were identified by autoradiography.

Miscellaneous

[32 P]P-enolpyruvate was prepared as described previously (Reizer *et al.*, 1984). For the calculation of intracellular concentrations, a specific internal volume of 3 μ l/mg of cell protein was used. Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. Plasmid DNA was isolated by the alkaline lysis method (Birnboim and Doly, 1979). For sequencing of double-stranded DNA, plasmid DNA was further purified by one or two polyethylene glycol precipitation steps and additional phenol/chloroform extraction, followed by ethanol precipitation and denaturation by alkali treatment (Sambrook *et al.*, 1989).

Materials

[carboxyl- 14 C]Benzoic acid (50 mCi/mmol), methyl- α -D-[14 C]glucopyranoside (50 mCi/mmol), and D-glucose-1-[14 C]lactose (57 mCi/mmol) were obtained from the Radiochemical Centre (Amersham, United Kingdom). [14 C]Methyl- β -D-thiogalactopyranoside (50 mCi/mmol) was obtained from Du Pont-New England Nuclear. [3 H]Melibiose (3.2 Ci/mmol) was a generous gift of Dr. G. LeBlanc (Bassilana *et al.*, 1987).

RESULTS

Histidine Mutagenesis—The lactose transport protein (LacS) of *S. thermophilus* contains 11 histidine residues (circled in Fig. 1A), of which 5 are present in the carrier domain and 6 in the IIA protein domain. Each of the histidine residues was replaced by site-directed mutagenesis with glutamine or arginine (Table II). Glutamine and arginine were chosen as replacements on the basis of amino acid similarity coefficients that were obtained from the main chain torsion angle distributions of highly resolved protein structures (Niefind and Schomburg, 1991). Plasmids with the histidine mutations were used to transform *E. coli* HB101, and transformants were streaked on lactose MacConkey agar plates. The *lac* phenotype of each of the mutants appeared to be indistinguishable from that of the wild-type strain, i.e. HB101/pSKE8 or HB101/pEKS8.

Lactose Transport by Histidine Mutants—Each of the plasmids bearing a mutation resulting in the replacement of a single histidine residue was used to transform *E. coli* DW2 (Δ lacZY). Lactose transport by DW2/pSKE8 (wild-type and histidine mutants) is shown in Fig. 2. With the exception of the H376Q (and to some extent, H155Q) mutant protein(s), the initial rates of uptake and the steady-state levels of lactose accumulation of the histidine mutants were similar to those of the wild-type. Similar results were obtained when transport

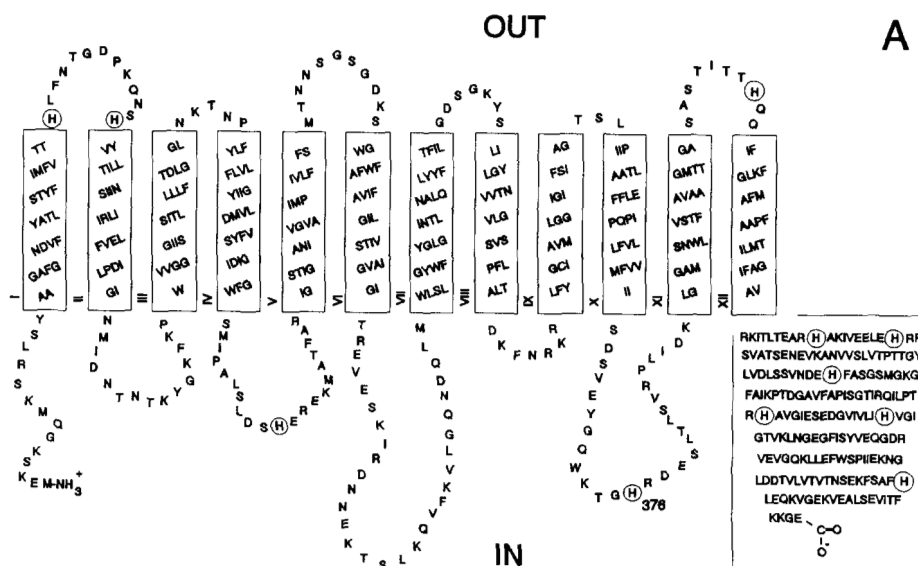
of TMG by the mutant proteins was assayed (data not shown). To ensure that secondary mutations did not contribute to the observed phenotype, the transport activity of each mutant was determined with two independent isolates. The data from a second set of mutants did not differ significantly from those presented in Fig. 2. To analyze the transport properties of each of the mutants in more detail, the affinity constant (K_T) and maximal rate of uptake (V_{max}) for lactose were determined (Table III). The data show that the H376Q mutant had a lower K_T and V_{max} for lactose, whereas the kinetic properties of the other mutants were not significantly affected.

Since LacS transports lactose as well as melibiose (α -galactoside), TMG, galactose, and other galactosides,³ the effects of the H376Q mutation on the uptake of these carbohydrates were compared (Fig. 3). To avoid metabolism of lactose and melibiose, transport studies were carried out with *E. coli* DW1 (Δ lacZY, Δ melAB)/pSKE8(wild type) and DW1/pSKE8(H376Q). The kinetic parameters for lactose uptake by *E. coli* DW1 bearing the wild-type or H376Q mutant proteins were similar to those obtained with *E. coli* DW2 bearing the corresponding transport protein (Table II). The data presented in Fig. 3 show that uptake of melibiose and TMG was markedly more affected by the H376Q mutation than uptake of lactose. In fact, the uptake rates were too low to determine accurately the kinetic parameters of the H376Q mutant (Fig. 3, B and C, insets). The K_T values for uptake of lactose, melibiose, and TMG by LacS(wild type) were 0.7, 0.53, and 0.27 mM, respectively. The V_{max} values for uptake of lactose, melibiose, and TMG were 270, 140, and 60 nmol/min/mg of protein, respectively.

In contrast to transport of lactose (Fig. 3A), TMG was not accumulated against a concentration gradient by the H376Q mutant (Fig. 3C). To establish whether this difference indeed reflected differences in the ability to accumulate various sugars or whether the apparent failure to accumulate TMG was due to higher efflux by passive and/or facilitated diffusion, a number of experiments were carried out. First, efflux of TMG was analyzed by diluting [14 C]TMG-loaded cells into buffer without TMG. No significant differences were observed between the wild-type and H376Q mutants (Fig. 4, ●). Second, the level of accumulation of TMG by the wild-type and H376Q proteins was determined over a wide range of concentrations (5–165 μ M) and at different pH values (5.5–8.0). If passive efflux of TMG, in combination with a reduced rate of uptake, affects the accumulation level, one might observe a significant

³ B. Poolman, unpublished data.

FIG. 1. A, secondary structure model of lactose transport protein of *S. thermophilus* based on hydrophathy plot of deduced amino acid sequence (Poolman *et al.*, 1989). Histidine residues are circled, and His-376 is also indicated by number. B, conserved residues in carrier domain of LacS and lactose, melibiose, and raffinose transport proteins of *E. coli*. The region corresponds to the loop that connects helices X and XI of LacS. Sources of sequences are as follows: *E. coli* (ec) RafB, Aslanidis *et al.* (1989); *K. pneumoniae* (kp), LacY, Mcmorrow *et al.* (1988); *E. coli* LacY, Büchel *et al.* (1980); *S. thermophilus* (st) LacS, Poolman *et al.* (1989); *L. bugarius* (lb) LacS, Poolman *et al.*⁴ and *E. coli* MelB, Yazyu *et al.* (1984). C, Conserved residues in IIA protein domain of LacS. The region shown corresponds to the proposed phosphorylation site of LacS. Sources of sequences are as follows (see also B): Crr, Nelson *et al.* (1983); NagE, Rogers *et al.* (1988); BglS (also indicated as BglF), Schnetz *et al.* (1987); *Streptococcus mutans* (sm) ScrA, Sato *et al.* (1989); and *B. subtilis* (bs) PtsG, Gonzy-Tréboul *et al.* (1989) and Sutrina *et al.* (1990). Residues corresponding to His-537 and His-552 in the lactose transport protein of *S. thermophilus* are indicated by arrows 1 and 2, respectively. Shaded arrows indicate residues that are conserved in the PTS proteins, but not in LacS.



Conserved residues in carrier domain of LacS

		1	2	3	4
RafB _{ec}	H325/E328	T	M	T	E
LacY _{kp}	H327/E330	S	A	V	E
LacY _{ec}	H322/E325	S	A	L	E
LacS _{st}	H376/E379	D	S	V	E
LacS _{lb}	H369/E372	D	A	V	E
MelB _{ec}	E361	D	I	V	D

Conserved residues in the IIA domain of LacS

		1	2
LacS _{st}	H537/H552	T	R
LacS _{lb}	H530/H545	T	R
Crr	H76/H91	T	N
NagE _{ec}	H554/H569	T	N
BglS _{ec}	H532/H547	T	L
ScrA _{sm}	H571/H586	T	G
PtsG _{bs}	H68/H83	T	K

concentration gradient ($[TMG]_{in} > [TMG]_{out}$) at low concentrations only. Some accumulation (2–3-fold) of TMG was observed at concentrations $<50 \mu M$ and at pH 5.5–6.0 (data not shown); the level of TMG accumulation by the wild-type strain also decreased with increasing TMG concentration. In conclusion, the data suggest that the H376Q mutation affects sugar recognition by LacS as well as the coupling between galactoside and proton transport.

To assess the sugar recognition properties of the wild-type and H376Q proteins in more detail, the effect of an 85-fold excess of unlabeled sugar on uptake of lactose was determined. The data presented in Table IV show that the galactosides lactose, melibiose, TMG, β -D-galactopyranosyl-1-thio- β -D-galactopyranoside, isopropyl-1-thio- β -D-galactopyranoside, and galactose inhibited uptake of [^{14}C]lactose (47 μM final concentration), whereas maltose (α -glucoside) and sucrose (fructofuranoside) did not. The pattern of inhibition of lactose uptake by the unlabeled sugars does not indicate major differences in sugar recognition of the wild-type and H376Q

transport proteins, although raffinose (trisaccharide/galactoside) was a more effective inhibitor of LacS(H376Q) compared with LacS(wild type).

The data presented above describe the effect of H376Q substitution on uphill (Δp -driven) transport by the lactose carrier protein. Under these conditions, a complete translocation cycle includes binding and release of H^+ and galactoside as well as reorientation of the loaded and unloaded binding sites. Since LacS also catalyzes an exchange reaction involving only binding and release of galactoside and reorientation of loaded binding sites, nonequilibrium exchange of TMG and lactose by the wild-type and H376Q transport proteins were compared (Figs. 4 and 5). For the exchange reaction, cells were preloaded with [^{14}C]TMG or [^{14}C]lactose and, following treatment with azide/carbonyl cyanide *m*-chlorophenylhydrazine (see "Experimental Procedures"), diluted 500-fold into buffers containing unlabeled TMG (or lactose) at concentrations ranging from 0 to 20 mM. Since the rate of net galactoside transport was negligible in these "energy-poisoned" cells (inferred from uptake by unloaded cells) (data not shown),

⁴ B. Poolman, S. Yeast, and B. F. Schmidt, unpublished data.

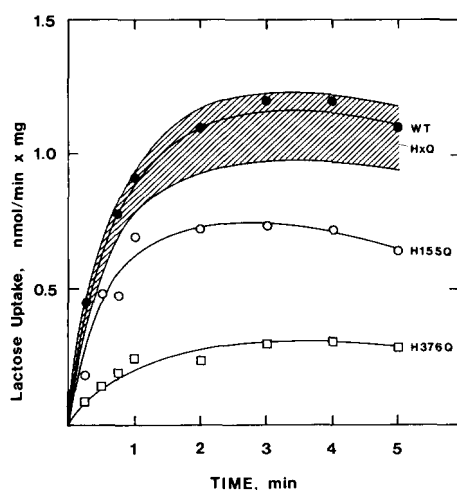


FIG. 2. Lactose uptake by *E. coli* DW2/pSKE8 wild-type and histidine LacS mutants. Cells were suspended in KPM buffer (pH 6.5) supplemented with 10 mM D-lithium lactate to a final protein concentration of 0.8 mg/ml. After 2 min of pre-energization, [14 C] lactose was added to a final concentration of 10 μ M, and uptake was assayed for different time intervals. Cells harvested in the stationary phase of growth were used; the assay temperature was 30 $^{\circ}$ C. HxQ, histidine mutants except H155Q and H376Q. \bullet , wild-type (WT) LacS; \circ , H155Q mutant; \square , H376Q mutants. The shaded area denotes the results obtained with all other histidine mutants of LacS.

TABLE III

Kinetic parameters of lactose transport by wild-type and histidine LacS mutants

Experimental conditions were similar to those described in the legend to Fig. 3, except that lactose concentrations ranged from 10 μ M to 1.6 mM.

Mutation	K_T	V_{max}
	mM	nmol/min/mg
Wild type	0.8	210
H39Q	1.0	163
H51Q	1.2	180
H155Q	1.1	215
H376Q	0.27	40
H422Q	1.1	214
H457Q	1.4	235
H466Q	0.6	150
H502Q	0.8	160
H537R	0.9	170
H552Q	1.0	174
H612Q	0.9	180

the observed isotope exit reaction reflects the real exchange of galactoside. Fig. 4 shows that initially the release of [14 C] TMG from the cell was monoexponential with a slope that depends upon the external TMG concentration. The exchange reaction catalyzed by the H376Q mutant was somewhat slower than that of the wild-type strain (the K_T values for external TMG were very similar, i.e. between 0.6 and 1.0 mM). This can be seen more readily from the kinetic analysis of lactose_{in}/lactose_{out} exchange (Fig. 5). At an internal lactose concentration of 4.5 mM (see legend to Fig. 5), the exchange kinetics yielded K_T values for external lactose of \sim 10 mM, which is about an order of magnitude higher than that of Δ p-driven lactose uptake (Fig. 3 and Table III).

Role of IIA Protein Domain—The homology between the IIA protein domain of LacS and the IIA protein (domain) of the glucose, *N*-acetylglucosamine, β -glucoside, and sucrose PTSs (Fig. 1C) suggests that Enzyme I and HPr can catalyze P-enolpyruvate-dependent phosphorylation of His-552 of the lactose transport protein. The data presented in Fig. 2 and

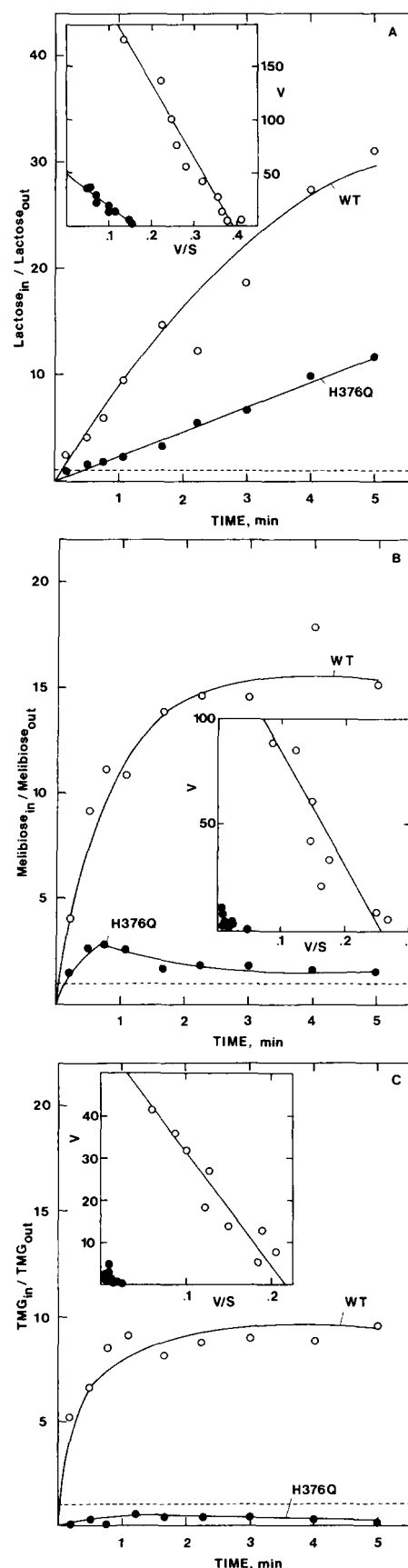


FIG. 3. Uptake of lactose, melibiose, and TMG by *E. coli* DW1/pSKE8 LacS(wild type) and LacS(H376Q). Experimental conditions were the same as described in the legend to Fig. 2, except that for the time course of galactoside uptake, cells harvested in the exponential phase of growth were used; [14 C]lactose, [3 H]melibiose,

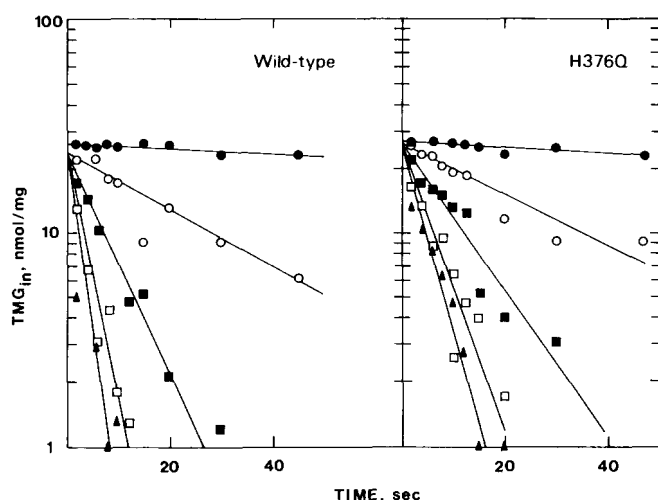


FIG. 4. Nonequilibrium exchange of TMG by *E. coli* DW2/pSKE8 LacS(wild type) and LacS(H376Q). Cells were preloaded (equilibrated) with 9 mM [14 C]TMG as described under "Experimental Procedures." Equilibration of TMG was confirmed from the estimated amount of [14 C]TMG taken up (expressed as nanomoles/milligram of protein) assuming a specific internal volume of 3 μ l/mg of protein. Exit of [14 C]TMG was determined following 500-fold dilution of a concentrated cell suspension (60–80 mg of protein/ml) into KPM buffer (pH 6.5) containing no TMG (●) efflux or 0.1 (○), 0.4 (■), 2 (□), or (▲) 10 mM TMG (nonequilibrium exchange). The assay temperature was 30 °C.

TABLE IV

Substrate specificity of LacS(wild type) and LacS(H376Q)

Lactose uptake was assayed at different time intervals between 0 and 90 s essentially as described in the legend to Fig. 2. Cells harvested in the stationary phase of growth were used; the assay temperature was 30 °C; the final [13 C]lactose concentration was 47 μ M. The sugars indicated were added to a final concentration of 4 mM (85-fold excess).

Sugar excess	LacS(wild type)		LacS(H376Q)	
	V_{Lac}	V_{Lac}	V_{Lac}	V_{Lac}
	nmol/min/mg	%	nmol/min/mg	%
No additions	10.8	(100)	1.3	(100)
Lactose	0.7	6	<0.1	<7
Melibiose	0.7	6	<0.1	<7
TMG	0.3	3	<0.1	<7
TDG ^a	0.4	4	<0.1	<7
Galactose	0.4	4	<0.1	<7
Maltose	9.5	88	0.9	69
Sucrose	10.7	99	1.1	85
IPTG	0.3	3	<0.1	<7
Raffinose	9.0	83	0.5	38

^a TDG, β -D-galactopyranosyl-1-thio- β -D-galactopyranoside; IPTG, isopropyl-1-thio- β -D-galactopyranoside.

Table III, on the other hand, indicate that the H552Q mutation does not affect transport activity. This suggests that phosphorylation of His-552 does not occur or that phosphorylation of the protein is not essential for transport activity. To assess the functional activities of the IIA protein domain of LacS, several approaches were undertaken. First, Δ p-driven uptake and exchange of lactose by DW2/pEKS8 and PPA209/pEKS8 (*ptsI*⁻) were compared. The results obtained with the

and [14 C]TMG were added to final concentrations of 45, 44, and 43 μ M, respectively; and the assay temperature was 37 °C. For the kinetics of galactoside uptake (*insets*), cells harvested in the stationary phase of growth were used. Initial rates of uptake were estimated from uptake after 10 s (in triplicate) at galactoside concentrations varying from 10 μ M to 1 mM. The assay temperature was 37 °C; the final protein concentrations were between 0.8 and 1.2 mg/ml. WT, wild type.

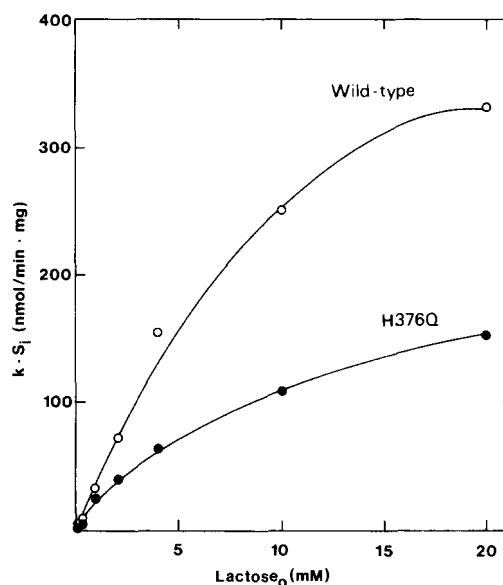


FIG. 5. Nonequilibrium exchange of lactose by *E. coli* DW2/pSKE8 LacS(wild type) and LacS(H376Q). Cells were loaded in the presence of 2.9 mM [14 C]lactose as described under "Experimental Procedures." From the amount of [14 C]lactose taken up (expressed as nanomoles/milligram of protein) and using a specific internal volume of 3 μ l/mg of protein, the final internal lactose concentrations were estimated to be 4.5 and 4.8 mM for *E. coli* DW2/pSKE8 LacS(wild type) and LacS(H376Q), respectively. Exit of [14 C]lactose was determined following 500-fold dilution of a concentrated cell suspension (~80 mg of protein/ml) into KPM buffer (pH 6.5) containing no lactose (efflux) or 0.1, 0.2, 0.4, 1.0, 2.0, 4.0, 10, or 20 mM lactose (nonequilibrium exchange). The release of [14 C]lactose from the cell was monoexponential, and first-order rate constants ($k = \ln 2/t^{1/2}$) could be determined. The initial rates (V) of exit were obtained by multiplying k with the internal lactose concentration ($V = k \times S_i$). The assay temperature was 30 °C.

ptsI mutant were similar to those of the wild-type mutant (data not shown). Second, manipulation of the level of PTS phosphoprotein intermediates by addition of nonmetabolizable sugar analog methyl- α -D-glucopyranoside and 2-deoxyglucose (Nelson *et al.*, 1986) did not affect the lactose transport activities. Third, pEKS8 (*lacS*⁺) was unable to complement *E. coli* LM1 (*crr*⁻, *nagE*) with respect to transport of α -methylglucoside. In accordance with previous observations (Vogler *et al.*, 1988), pAVL1 containing *nagE* was able to restore the uptake of α -methylglucoside in *E. coli* LM1 to ~20% of the wild-type activity (strain LR2-167). Fourth, by examining the sequences of the IIA protein(s) (domains) of the lactose transport proteins of *S. thermophilus* and *L. bulgaricus* and the PTS proteins indicated in Fig. 1C, two positions were identified at which the lactose transport proteins differed significantly from the PTS proteins, *i.e.* Ile-548 and Gly-556. Three residues are close to proposed phosphorylation site residue (His-552); and the crystal structure of the IIA^{Glc} protein domain of the glucose PTS of *B. subtilis* indicates that the equivalent residues, *i.e.* Glu-79 and Asp-87, can assume a role in recognition of the interactive PTS proteins (the IIB protein domain or HPr) and facilitate the phosphoryl transfer reaction (Liau *et al.*, 1991). The double mutant LacS(I548E/G556D) was constructed, but the I548E/G556D mutations did not affect lactose transport activity (data not shown).

Phosphorylation of Lactose Transport Protein—To establish whether LacS could be phosphorylated by HPr(His~P), inside-out membrane vesicles of *E. coli* T184/pKK223-3 (*lacS*⁻, vector control) and T184/pEKS8 (*lacS*⁺) and various mutants

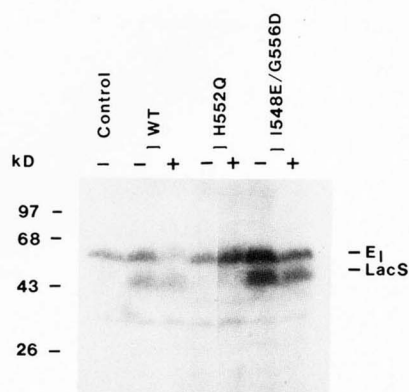


FIG. 6. *In vitro* phosphorylation of wild type and mutants LacS proteins. Phosphorylation of LacS was achieved as described under "Experimental Procedures." The *minus* and *plus* signs refer to the absence and presence of 1 mM 2-deoxyglucose in the phosphorylation assay. The positions of phosphorylated Enzyme I (E_I) and LacS are indicated. The first lane shows the results of a control phosphorylation reaction using membranes that do not bear LacS. WT, wild type.

(see "Experimental Procedures") were isolated. The results shown in Fig. 6 indicate that the wild-type mutant as well as the I548E/G556D mutant were readily phosphorylated by [32 P]P-enolpyruvate in the presence of purified Enzyme I and HPr isolated from *B. subtilis*. By contrast, no phosphorylation of the mutant H552Q was detected. The phosphorylation of the proteins was not significantly affected by methyl- α -D-glucopyranoside. Surprisingly, but in accordance with the data presented above, phosphorylation of LacS could not be detected with Enzyme I and HPr isolated from *E. coli* (data not shown). Altogether, these results indicate that PTS-catalyzed phosphorylation of His-552 of LacS can occur, but that it does not serve an essential functional role in the transport of lactose under the conditions employed in this study.

DISCUSSION

On the basis of hydropathy analysis and a comparison of the primary structures of LacS and MelB, a secondary structure model of the lactose transport protein is proposed (Fig. 1A). The polypeptide is organized into 12 hydrophobic transmembrane α -helical domains that are connected alternately by periplasmic (OUT) and cytoplasmic (IN) hydrophilic charged segments. The topology of the periplasmic loops that connect helices I and II, III and IV, and V and VI as well as the cytoplasmic loop that connects helices VI–VII has been confirmed by analyzing *lacS*-alkaline phosphatase gene (*phoA*) fusions (Manoil and Beckwith, 1986).⁵ The IIA protein domain (boxed sequence in Fig. 1A) is proposed to be on the cytoplasmic side of the membrane. LacS contains 11 histidine residues (circled in Fig. 1A), of which 5 are present in the carrier domain and 6 in the IIA protein domain. None of the histidine residues of LacS are conserved in the homologous melibiose carrier protein (MelB). Although LacS and the lactose carrier protein (LacY) of *E. coli* are not homologous (Poolman *et al.*, 1989), a region similar to the proposed catalytic site of LacY was identified in LacS (Fig. 1B). The conserved charged residues in the regions flanking His-376 of LacS and His-322 of LacY (indicated by arrows) are also conserved in the LacS protein of *L. bulgaricus*, the LacY protein of *K. pneumoniae*, and the raffinose transport protein (RafB) of *E. coli* (Fig. 1B). His-322 (arrow 3) and Glu-325 (arrow 4), together with Arg-302 of LacY, have been proposed

to form a H^+ relay system that could function as the chemical pathway for H^+ movement through the lactose carrier (Carrasco *et al.*, 1986, 1989; Püttner *et al.*, 1986, 1989; Menick *et al.*, 1987; Lee *et al.*, 1989). Although evidence against this proposal has been presented (King and Wilson, 1989a, 1989b; Franco and Brooker, 1991), it is evident that this region is crucial for energy transduction by the carrier protein. Mutations at position 322 in LacY also affect the sugar recognition properties of LacY (Franco *et al.*, 1989; Collins *et al.*, 1989). Indeed, some properties of the LacY(H322) mutants resemble those of LacS(H376Q). For instance, active accumulation of lactose, melibiose, and TMG is highly compromised in cells bearing the H376Q protein. Furthermore, the affinity for galactosides (lactose) is altered by the H376Q substitution. The histidine residue corresponding to His-376 in LacS and His-322 in LacY is not conserved in MelB. Recent experiments in which the histidine residues of MelB were replaced with arginine residues indicated that only His-94 is important for transport activity (Pourcher *et al.*, 1990a). The H94R mutation caused complete loss of sugar binding and transport, whereas *melB* was expressed normally.

Interestingly, the residue corresponding to Glu-325 of LacY (Fig. 1B, arrow 4) is conserved in the lactose transport proteins of *E. coli*, *K. pneumoniae*, *S. thermophilus*, and *L. bulgaricus*; the raffinose transport protein of *E. coli*; and the melibiose carrier of *E. coli*. Substitution of Glu-325 in LacY leads to a transport protein that does not catalyze lactose/ H^+ symport or lactose efflux, but catalyzes exchange and counterflow at normal rates (Carrasco *et al.*, 1986). Similarly, substitution of the corresponding residue in MelB (Glu-361) for glycine, aspartate, or alanine affects sugar and cation translocation, but not recognition of the substrates (Pourcher *et al.*, 1990b).

Mutations of the conserved lysine (Fig. 1B, arrow 2) have been isolated for LacY after selection for enhanced recognition of maltose (α -glucoside) and resistance for TDG (Collins *et al.*, 1989). The LacY(K319N) mutant protein exhibits diminished recognition of β -galactosides and β -glucosides. The conserved acidic residue corresponding to arrow 1 (Fig. 1B) has not been investigated in any of the transport proteins. Overall, comparison of the sequences presented in Fig. 1B and the data obtained from mutagenesis experiments suggests that the region connecting helices X and XI of LacS is important for substrate recognition.

A difference worth noting between the LacS and LacY regions is the higher polarity of the residues around His-376 (LacS) in comparison to the region around His-322 (LacY). As a consequence, hydropathy analysis predicts the His-322 region of LacY to be in the membrane, whereas the His-376 region of LacS is predicted to be in a cytoplasmic loop. Nevertheless, the local environment of the important residues in both protein segments might be similar if the regions are located near the head groups of the lipid bilayer.

The effect of the H376Q substitution on galactoside transport by LacS has been analyzed by measuring Δp -driven uptake and exchange under nonequilibrium conditions. Assuming that the V_{max} of the exchange reaction reflects the level of expression of the lactose transport protein, the effect of the H376Q mutation on the k_{cat} of Δp -driven uptake is ~ 2 -fold smaller than indicated by the V_{max} values for uptake of lactose, melibiose, and TMG (Fig. 3). The observation that Δp -driven transport of galactosides is more severely affected by the His-376 mutation than the exchange reaction suggests that the histidine at position 376 is important for energy transduction, *i.e.* coupled movement of galactosides and protons. The results also indicate that His-376 is not obligatorily

⁵ V. Swarte and B. Poolman, unpublished data.

required for the active accumulation of galactosides.

The *in vitro* phosphorylation assays indicated that LacS can be phosphorylated by HPr(His~P) of *B. subtilis* and that His-552 in the IIA protein domain is most likely the phosphorylation site. Nevertheless, the *in vivo* transport experiments that were performed in *E. coli* bearing LacS did not provide an indication regarding the functional and/or regulatory role(s) of the phosphorylation reaction. Although *B. subtilis* IIA^{Glc} can readily replace *E. coli* IIA^{Glc} with respect to sugar transport and regulation (Reizer *et al.*, 1992), it is possible that the IIA protein domain of LacS is a poor phosphoryl acceptor and/or phosphoryl donor of the *E. coli* HPr(His~P) and/or the IIB^{Glc} domain, respectively. Consequently, discerning the role of LacS phosphorylation may require the use of PTS constituents derived from homologous systems (or other Gram-positive bacteria) rather than the heterologous system used in the *in vivo* studies described here. In addition, we cannot exclude the possibility that phosphorylation of the IIA protein domain of LacS does not play a direct role in the translocation of lactose across the membrane, but rather functions in P-enolpyruvate-dependent and PTS-catalyzed phosphorylation of the intracellularly formed glucose following hydrolysis of the incoming lactose. Guided by these suppositions, a study is now underway to examine the role of LacS phosphorylation in *S. thermophilus*.

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